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SHORT REVIEW

Evidence for Direct Roles of Calcium in Photosynthesis

Jerry J. Brand[1] and David W. Becker[2]

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Abstract

Calcium may function directly in several aspects of photosynthesis. It appears to modulate activity of the phosphatase enzymes in the carbon reduction cycle and also to regulate chloroplast NAD^+ kinase activity through a calmodulin-like protein. Some evidence supports a calcium function in the water-splitting complex, and other evidence indicates a reaction center function in photosystem II. Calcium in reaction center II may be tightly bound in chloroplasts and weakly bound in blue-green algal thylakoids. Free calcium concentration in stroma is probably $<10^{-6}$ M, although the absolute concentration is not yet known. Intrathylakoid calcium content is likely very high. Stromal calcium may regulate several enzyme activities, while intrathylakoid calcium may promote photosystem II constitutively. Results to date demonstrate the need for more attention to cation composition in studies of both light and dark reactions of photosynthesis, and the need to identify free calcium levels in chloroplasts.

Key Words: Calcium in blue-green algae; calcium in chloroplasts; calcium in photosynthesis; calcium levels in plants; cyanobacterial photosynthesis; photosynthesis regulation.

Introduction

Calcium performs a number of regulatory roles within living cells [see Cheung (1982) and Scarpa and Carafoli (1978) for general reviews], and the importance of Ca^{2+} for plant cells is now becoming recognized (Roux and Slocum, 1982). We will not attempt here to survey these diverse functions, but several general observations are of direct interest. Most well-documented regulatory activities of Ca^{2+} occur within the cytoplasmic matrix of eukaryotic cells. In animal cells the free Ca^{2+} concentration is maintained at 10^{-6} to 10^{-8} M within the cytoplasm, a concentration range which best affords

[1]Department of Botany, University of Texas, Austin, Texas 78712.
[2]Agronomy Department, University of Kentucky, Lexington, Kentucky 40506.

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metabolic regulation. Cytoplasmic free Ca^{2+} levels in *Chara* and *Nitella,* two green algae, are also micromolar to submicromolar (Williamson and Ashley, 1982), and it is likely that plant cells in general have similar cytoplasmic free Ca^{2+} levels. Both mitochondria (Earnshaw *et al.,* 1973) and chloroplasts (O'Keefe and Dilley, 1977) accumulate Ca^{2+} at a much higher mean concentration, although this large amount is not all soluble nor is it uniformly distributed. This apparently excessive accumulation of Ca^{2+} is often proposed to represent only a source and sink for regulation of cytoplasmic Ca^{2+}. Yet several photosynthetic functions are influenced specifically by Ca^{2+}.

Prokaryotes have no known requirement for Ca^{2+} except for endospore formation (Rosen, 1982). The prokaryotic blue-green algae (cyanobacteria), however, provide strong evidence for a Ca^{2+} function in photosystem II (see below). The green and purple photosynthetic bacteria perform an anoxygenic photosynthesis with only one photosystem. No clear evidence for a Ca^{2+} function has yet been demonstrated in these organisms, although isolated chromatophores of *Chromatium vinosum, Rhodospirillum rubrum* (Davidson and Knaff, 1981), and *Rhodopseudomonas capsulata* (Jasper and Silver, 1978) exhibit light-dependent Ca^{2+} uptake in exchange for protons. This review will consider Ca^{2+} function only in oxygenic photosynthesis.

Calcium Content and Flux in Chloroplasts

Early determinations of the ion distribution in plants indicated that a large proportion of leaf Ca^{2+} resides in chloroplasts. These studies used aqueous (Neish, 1939) and nonaqueous (Stocking and Ongun, 1962) isolation techniques to minimize ion leakage during chloroplast isolation. More recent analyses have revealed amounts of Ca^{2+} which would correspond to a mean concentration of 15–25 mM provided that all were in solution (Larkum, 1968; Nobel, 1969; O'Keefe and Dilley, 1977; Yamagishi *et al.,* 1981).

The free Ca^{2+} concentration in the stroma is apparently very low; otherwise phosphate would precipitate and photophosphorylation would be inhibited. Thus most chloroplast Ca^{2+} must be bound to membranes, associated with macromolecules, precipitated in some manner, and/or sequestered within intrathylakoid spaces. From experiments designed to measure ionophore-mediated leakage of free Ca^{2+} into calcium-free medium, Miginiac-Maslow and Hoarau (1977) concluded that virtually no free Ca^{2+} exists in chloroplasts. Yamagishi *et al.* (1981) measured thermodynamic activities of K^+, Na^+, Ca^{2+}, and Mg^{2+} in intact chloroplasts from the marine siphonaceous green alga, *Bryopsis.* Free Ca^{2+} concentration was 0.6 mM, representing less than 5% of total chloroplast calcium. The methods employed could not easily

distinguish between stroma and intrathylakoid Ca^{2+}, so it is possible that stroma levels were very much lower.

Assuming that the free Ca^{2+} content of stroma is very low, where does the large majority of chloroplast Ca^{2+} reside? There is evidence which indicates that it accumulates in intrathylakoid spaces (Nobel *et al.*, 1966; O'Keefe and Dilley, 1977). Calcium also will bind to thylakoid membranes where it elicits several responses, but neutron activation analysis indicates that the predominant cation species comprising the diffuse double layer of chloroplast membranes normally is Mg^{2+} (Nakatani *et al.*, 1979). Other evidence (Cournier *et al.*, 1982) suggests that chloroplast Ca^{2+} is bound mostly to carboxyl groups on membrane proteins, and significant amounts may be bound to sites buried within the membranes (Prochaska and Gross, 1977). Insoluble Ca^{2+} precipitates may occur within chloroplasts (Nobel and Murakami, 1967), although no recent evidence has confirmed this observation.

The high Ca^{2+} content of chloroplasts with respect to cytoplasm suggests the presence of Ca^{2+} pumps. Cation transport across the envelope of intact chloroplasts is generally reported to be low (Heber and Heldt, 1981), but relatively high rates of light-dependent Ca^{2+} uptake occur in intact wheat and spinach chloroplasts (Muto *et al.*, 1982). The uptake occurs via an H^+/Ca^{2+} antiport, is noncompetitive with K^+ or Mg^{2+}, and demonstrates a K_m only slightly higher than cytoplasmic Ca^{2+} concentrations.

Previously ATP-dependent Ca^{2+} uptake had been observed in isolated chloroplasts (Nobel and Packer, 1964; Dilley and Vernon, 1965; Nobel and Packer, 1965), but these earlier methods would not have produced intact chloroplasts, so the uptake may have involved primarily thylakoid rather than envelope membranes.

Calcium and other cations exchange with H^+ during coupled electron transport. Although the membrane transport system has a higher affinity for Ca^{2+} than Mg^{2+} (Hind *et al.*, 1974), several lines of evidence indicate that Mg^{2+}/H^+ exchange predominates in intact chloroplasts (Barber *et al.*, 1974; Miginiac-Maslow and Hoarau, 1977). It appears that Ca^{2+} uptake across the chloroplast envelope contributes to the maintenance of low cytoplasmic Ca^{2+}. Uptake by the thylakoids in turn may help to prevent accumulation in the stroma.

Calcium Effects on Stroma Enzymes

Portis and Heldt (1976) demonstrated that Ca^{2+} may inhibit CO_2 fixation in intact chloroplasts. Charles and Halliwell (1980) determined that

Ca^{2+} inhibits fructose-1,6-bisphosphatase and that this inhibition is competitive with Mg^{2+}. Hertig and Wolosiuk (1980) also noted this inhibition, but observed in addition that activation of the enzyme by thioredoxin and substrate is greatly facilitated by the presence of Ca^{2+} (or Mn^{2+}). Sedoheptulose-1,7-biphosphatase responds similarly toward Ca^{2+}, both in terms of activation and inhibition Wolosiuk *et al.*, 1982). These two phosphatases are key regulatory components of the photosynthetic carbon reduction pathway of photosynthesis (Buchanan, 1980). Thus, Ca^{2+} may play a regulatory role in this cycle, a proposal which is strengthened by the observation (Charles and Halliwell, 1980) that Ca^{2+} inhibits phosphatase activity much more at pH 7 (approximate stroma pH in darkness) than at pH 8 (stroma pH during steady-state illumination). However, the K_I for Ca^{2+} inhibition was 40 μM, much higher than would be predicted from the presumed free Ca^{2+} concentration of the stroma. A recently developed method for assaying fructose-1,6-bisphosphatase activity under steady-state conditions of constant substrate and Mg^{2+} concentrations demonstrated rapid activation of the enzyme with no requirement for Ca^{2+} (Rosa, 1981). Thus, Ca^{2+} regulation of the dark reduction cycle of photosynthesis may include only an inhibitory role of the two bisphosphatases. In any case, conclusive demonstration requires that the effects be shown at *in vivo* concentrations of stroma Ca^{2+} and other regulatory components. This, in turn, requires that free Ca^{2+} levels be determined in the stroma.

Leaves of C_4 plants have very low levels of Ca^{2+}. This may be important for regulation of C_4 photosynthesis via regulation of pyrophosphatase (Gavalas and Manetas, 1980a) and PEP carboxylase (Gavalas and Manetas, 1980b) activities. As with the Ca^{2+} effects on other photosynthetic carbon reduction enzymes it will be important to correlate K_I of the Ca^{2+} inhibitions with the *in vivo* concentrations of stromal free Ca^{2+}.

Plant NAD^+ kinase occurs both in the cytoplasm and in chloroplasts of leaf cells. It is activated by an acidic, heat-stable protein (Muto and Miyachi, 1977) which requires Ca^{2+} for its regulatory effect (Anderson and Cormier, 1978). Authentic calmodulin substitutes for this regulatory protein. Recently Jarrett *et al.* (1982) and Muto (1982) independently isolated calmodulin-like proteins from pea and wheat chloroplasts, respectively. The amount isolated in each case was small, but large enough to account for NAD^+ kinase regulation within chloroplasts. Direct confirmation by amino acid sequencing of these proteins was not possible because of the small amounts isolated. Phenathiazine drugs inhibited the conversion of NAD^+ to $NADP^+$ in chloroplasts (Jarrett *et al.*, 1982), supporting the likelihood of a role for calmodulin in this process. Thus, Ca^{2+} may regulate $NADP^+$ availability in photosynthesis through calmodulin or a calmodulin-like protein. One must view results with phenathiazine drugs with caution, however, as these drugs exert generalized

effects on membranes (Corps *et al.*, 1982). Use of more specific calmodulin antagonists, such as the W compounds (Hidaka *et al.*, 1981), is necessary to confirm this (and other) implied calmodulin effects on photosynthesis.

Calcium Effects on Photosynthetic Activities of Chloroplast Thylakoid Membranes

Many photosynthetic studies of lamellar preparations from chloroplasts have demonstrated a mineral cation requirement for a particular activity. Examples of activities which show a response to Ca^{2+} include regulation of energy distribution between the two photosystems (Barber, 1982), thermal stabilization of the photosynthetic membranes (Weis, 1982), binding of coupling factor (Telfer *et al.*, 1980) and plastocyanin (Tamura *et al.*, 1981) to the membranes, thylakoid stacking (Izawa and Good, 1966; Smillie *et al.*, 1976), and stimulation of electron transport activities, particularly when an anionic electron donor or acceptor is used (Itoh, 1978; Rurainski and Mader, 1977). These effects are most often explained as a general salt bridge requirement, a membrane charge screening effect, or an ionic strength requirement, with Mg^{2+} usually proposed as the physiological ion of importance.

Evidence has been accumulating for several years which more specifically implicates Ca^{2+} directly in photosystem II electron transport. Barr *et al.* (1980) incubated chloroplast membranes with EGTA and observed effects on the light reactions. All photosystem II partial reactions were inhibited, including electron flow from diphenylcarbazide to silicomolybdate, indicating that the site of inhibition was near the reaction center. Exogenously added Ca^{2+} partially restored photosystem II activities. Phenathiazine drugs also inhibited photosystem II at a site near the reaction center (Barr *et al.*, 1982), suggesting the involvement of a Ca^{2+}-binding protein. Excess Ca^{2+} protected against the inhibitory effect of these compounds. The results were somewhat complicated since EGTA affects more than one site of electron transfer (Barr *et al.*, 1980) and since phenathiazine compounds also uncouple photophosphorylation at very low concentrations (Barr and Crane, 1982) and have generalized effects on membranes as indicated above.

Barr and Crane (1983) subsequently showed that acid treatment removed from thylakoids several proteins, among which, one, after some purification, gave enhanced restoration of photosystem II activity to acid-treated chloroplasts in the presence of Ca^{2+}. Partial electron-transport reactions suggested that the effect was near the reaction center. Barr *et al.* (1983), in a separate communication, identified a calcium-selective site of action between the two photosystems. This effect involved binding of Ca^{2+} to

(presumably) protein carboxyl groups. Prochaska and Gross (1977) had previously reported a Ca^{2+} binding site on Photosystem II particles.

EPR signal II_f is a measure of the oxidized form of a primary oxidant, Z, of photosystem II. Yerkes and Babcock (1981) observed a specific Ca^{2+} effect on this signal (enhanced decay kinetics) in addition to a general cation effect.

Other evidence suggests a role for Ca^{2+} in the oxygen-evolving part of photosystem II. Yamashita and Tomita (1974) demonstrated that both Mn^{2+} and Ca^{2+} were required for complete light reactivation of Tris- or Tris-acetone-washed chloroplasts, while Mg^{2+} was not effective. If light reactivation occurred prior to treatment with EDTA, the system exhibited stability against this divalent cation chelator, indicating that the cations may have been incorporated into an inaccessible site during light reactivation. Yamashita and Tomita (1976) later showed that the Ca^{2+} effect required light and suggested that it may be associated with an energy coupling process. Recently Ono and Inoue (1983) reported dual requirements for Mn^{2+} and Ca^{2+} for the photoactivation of water oxidation in intact chloroplasts isolated from leaves grown under a flashing light regime. Low concentrations of Mn^{2+} were effective, but higher concentrations inhibited the photoactivation. Magnesium and Sr^{2+} also inhibited, but in each case addition of Ca^{2+} abolished the inhibition. When the chloroplasts were treated with EGTA prior to reactivation attempts, Mn^{2+} alone could not effect recovery. By kinetic analysis these workers showed that higher concentrations of Ca^{2+} competitively inhibited at Mn^{2+} binding sites and high concentrations of Mn^{2+}, Mg^{2+}, or Sr^{2+} resulted in competitive inhibition at Ca^{2+} binding sites.

Marine photosynthetic organisms may have a more pronounced Ca^{2+} requirement than land plants. Burris and Black (1983) demonstrated an inhibition by the phenathiazine drug, trifluoperazine, of photosynthetic O_2 evolution in both isolated and symbiotically adapted zooxanthellae, although the site and mechanism of inhibition was not characterized. Critchley *et al.* (1982) demonstrated a rather specific Ca^{2+} enhancement of activity in addition to the pronounced Cl^- requirement for photosystem II in thylakoids isolated from mangrove chloroplasts.

Calcium in Photosystem II of Blue-Green Algae

Although they lack true chloroplasts and have a different accessory pigment system from that of most chloroplasts, blue-green algae (cyanobacteria) perform photosynthesis via essentially the same mechanism as do green plants (Ho and Krogmann, 1982). Yet, in contrast to those from green plants, active photosynthetic membranes from blue-green algae are difficult to

prepare. Photosystem II activities and noncyclic photophosphorylation are particularly labile. Fredricks and Jagendorf (1964) were able to demonstrate some photosynthetic O_2 evolution in membranes from *Anacystis* only when a divalent cation (Ca^{2+} worked best) was present during assays. Another factor, presumably a protein, which was recovered from the broken cell extracts further stimulated O_2 evolution. Membranes prepared from *Nostoc* (McSwain *et al.*, 1976), *Tolypothrix, Anacystis, Anabaena* (Black *et al.*, 1963), and *Anabaena* (Susor and Krogmann, 1964) retained O_2 evolution activity in the presence of divalent cations. The *Anabaena* preparations retained high photosystem II activities. More recently active membrane preparations were obtained from *Phormidium* by first treating cells with lysozyme to effect wall weakening, then gently breaking under controlled conditions (Binder *et al.*, 1976). High oxygen evolution rates occurred only when osmotic support and divalent cations were present during and subsequent to cell rupture. Membrane vesicles from *Spirulina* showed high rates of ferricyanide-mediated O_2 evolution only in the presence of cations (DeRoo and Yocum, 1981). Trivalent cations worked better than divalents in this sonicated preparation, and a membrane charge screening effect was probably involved. In these and most other early experiments with blue-green algal membranes Mg^{2+} was believed to be the ion of physiological importance since Ca^{2+} was known to inhibit photophosphorylation and aggregate membrane preparations.

The first evidence that Ca^{2+} performs a specific role in photosystem II of blue-green algae was obtained with *Phormidium.* Membrane vesicles were capable of some O_2 evolution even when prepared without osmotic support, but Ca^{2+} was obligately required during assays (Piccioni and Mauzerall, 1976). Maximum rates were low and the ferricyanide reduction accompanying O_2 evolution was partially insensitive to DCMU (3-[3,4-dichlorophenyl]1,1-dimethylurea) but Ca^{2+} stimulated the rate some twentyfold over that in the presence of Mg^{2+}. Calcium increased both the number of active photosynthetic units and the turnover rate (Piccioni and Mauzerall, 1978a). Although absolute rates of O_2 evolution varied with the Hill acceptor employed and the redox potential at which the membrane suspension was poised, activities showed a specific Ca^{2+} effect beyond a general cation effect (Piccioni and Mauzerall, 1978b).

Work in this laboratory has characterized a specific effect of Ca^{2+} on photosynthetic membranes from *Anacystis.* High rates of O_2 evolution accompanying ferricyanide or benzoquinone reduction were obtained in membranes prepared from cells broken with a French pressure cell (Brand, 1979). Osmotic support was required throughout the preparation and Ca^{2+} was required during cell breakage. When assays were then performed in the presence of Mg^{2+}, both cyclic and noncyclic photophosphorylation occurred as

well (Yu and Brand, 1980). These results were interpreted to indicate that Mg^{2+} functions on the outer surface of thylakoid membranes to facilitate photophosphorylation, while Ca^{2+} functions on the inner surface to maintain photosystem II activity.

England and Evans (1981) prepared photosystem II particles from *Anacystis* and *Phormidium* by detergent fractionation. Oxygen evolution activity was retained in these particles only when Ca^{2+} was present during the initial steps of the preparation. Several polypeptides were lost when Ca^{2+} was not present, which correlated with a marked decline in photosystem II activity (England and Evans, 1983). The Ca^{2+} requirement was retained in photosystem II reactions which bypass the oxygen-evolving site, indicating that the site of action is near the reaction center. Chlorpromazine inhibited activity in these active photosystem II particles, suggesting the presence of a Ca^{2+}-binding protein, although a definitive statement cannot be made in view of the known general effects of phenathiazines.

The Ca^{2+} requirement for photosystem II can be demonstrated in whole cells of *Anacystis*. When cells are incubated in a medium devoid of Ca^{2+} and containing a divalent cation chelator, a pronounced decline in photosynthesis occurs (Becker and Brand, 1982). This effect occurs only if Na^+ is also absent from the nutrient medium. Growth stops, since *Anacystis* is an obligate autotroph, but if Ca^{2+} or Na^+ is introduced into the culture medium the cells rapidly regain photosynthetic competency and growth subsequently resumes. This *in vivo* effect on photosynthesis occurs in photosystem II, since O_2 evolution accompanying benzoquinone reduction is inhibited by Ca^{2+}/Na^+ depletion while photosystem I partial reactions are not. Of a large number of mono-, di-, and trivalent cations tried (including K^+ and Mg^{2+}), only Ca^{2+} and Na^+ demonstrate this effect. This unusual and interesting specific substitution of Na^+ for Ca^{2+} may well be a function of their nearly identical ionic radii. In the experiments described below, using a medium without a Na^+ component, control cells were incubated in the presence of Ca^{2+} and exhibited normal growth and photosynthetic activities for the duration of each experiment. Effects of Ca^{2+} depletion were observed on samples incubated in the same medium but without Ca^{2+}.

Measurements of fluorescence in control and Ca^{2+}-depleted cells localized the site of function to the vicinity of the reaction center (Brand *et al.*, 1983). Variable fluorescence was eliminated and the steady-state level remained near F_0 in depleted cells, when measured either at cell growth temperature or at 77°K. Neither DCMU nor hydroxylamine had any effect on fluorescence yield in the depleted cells. Millisecond delayed light emission was lost in parallel with decreases in variable fluorescence and photosystem II photochemistry. Addition of Ca^{2+} to the culture medium restored all of these parameters in parallel.

Diminished electron flow from diphenylcarbazide to silicomolybdate occurs in membranes isolated from Ca^{2+}-depleted cells, and this loss parallels loss in photosystem II activity in whole cells (Becker and Brand, unpublished). EPR signal II is also diminished in Ca^{2+}-depleted cells and is restored when Ca^{2+} is added (Boska, Becker, Brand, and Sauer, unpublished). Thus, the site of action is localized to either the reaction center or immediately to its oxidizing side.

Neither light absorption nor steady-state fluorescence spectral characteristics are significantly altered as photosystem II activity declines during Ca^{2+} depletion in intact *Anacystis* cells. As photosystem II activity approaches zero, however, fluorescence increases. When measured at 77°K this increase occurs as a broad peak centered near 660 nm and a larger peak at 681 nm (Mohanty, Brand, and Fork, unpublished). The increased fluorescence occurs when phycocyanin is excited, but not when chlorophyll is excited. The fluorescence intensity at these peaks continues to increase long after all photosystem II activity is lost due to Ca^{2+} depletion. These experiments demonstrate that a secondary effect of Ca^{2+} removal from *Anacystis* cells is energetic detachment of phycobilisomes from the photosynthetic membranes. The phycobilisomes apparently remain intact since the 681 nm fluorescence is likely an allophycocyanin emission (Glazer, 1982).

The disconnection of phycobilisomes in severely Ca^{2+}-depleted cells might suggest that photosystem II inhibition occurs by less efficient energy transfer to the reaction center. Yet, this is clearly not the case. The light saturation kinetics of photosystem II are identical in control cells and those in various stages of depletion (Becker and Brand, unpublished). Oxygen flash yields decline, indicating a decrease in the number of functional reaction centers II (Jack Myers, personal communication). Also, the maximum turnover rate (as determined by flash yield measurements) does not decline. Thus, the depletion affects photosystem II reaction centers on an all-or-nothing basis, rather than by a gradual decrease in rate of electron flow through active centers. A secondary and separate effect is the energetic detachment of phycobilisomes.

Not enough species of blue-green algae have been studied yet for a general conclusion, but those so far examined appear to require Ca^{2+} for photosystem II activity. At least in *agmenellum*, *anabaena*, and *fremyella*, Na^+ does not effectively substitute for Ca^{2+}.

Note Added in Proof

This survey was completed prior to publication of a number of reports which suggest that Ca^{2+} may relate to the function of a 23 KD protein on the oxidizing side of Photosystem II.

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